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Mechanism of Citrate Metabolism by an Oxaloacetate Decarboxylase-Deficient Mutant of *Lactococcus lactis* IL1403[▽]

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Citrate metabolism in resting cells of *Lactococcus lactis* IL1403(pFL3) results in the formation of two end products from the intermediate pyruvate, acetoin and acetate (A. M. Pudlik and J. S. Lolkema, J. Bacteriol. 193:706–714, 2011). Pyruvate is formed from citrate following uptake by the transporter CitP through the subsequent actions of citrate lyase and oxaloacetate decarboxylase. The present study describes the metabolic response of *L. lactis* when oxaloacetate accumulates in the cytoplasm. The oxaloacetate decarboxylase-deficient mutant ILCitM(pFL3) showed nearly identical rates of citrate consumption, but the end product profile in the presence of glucose shifted from mainly acetoin to only acetate. In addition, in contrast to the parental strain, the mutant strain did not generate proton motive force. Citrate consumption by the mutant strain was coupled to the excretion of oxaloacetate, with a yield of 80 to 85%. Following citrate consumption, oxaloacetate was slowly taken up by the cells and converted to pyruvate by a cryptic decarboxylase and, subsequently, to acetate. The transport of oxaloacetate is catalyzed by CitP. The parental strain IL1403(pFL3) containing CitP consumed oxaloacetate, while the original strain, IL1403, not containing CitP, did not. Moreover, oxaloacetate consumption was enhanced in the presence of L-lactate, indicating exchange between oxaloacetate and L-lactate catalyzed by CitP. Hence, when oxaloacetate inadvertently accumulates in the cytoplasm, the physiological response of *L. lactis* is to excrete oxaloacetate in exchange with citrate by an electroneutral mechanism catalyzed by CitP. Subsequently, in a second step, oxaloacetate is taken up by CitP and metabolized to pyruvate and acetate.

Citrate metabolism plays an important role in many food fermentation processes involving lactic acid bacteria (LAB) (11). Citrate is the precursor of carbon dioxide and the flavor compounds diacetyl and acetoin that contribute to the organoleptic properties of fermented foods. The flavor compounds are formed from the central metabolite pyruvate in the cytoplasm. Citrate metabolism feeds directly into the pyruvate pool (Fig. 1). Following uptake by the secondary citrate transporter CitP, citrate lyase (CL) converts citrate to acetate and oxaloacetate. Acetate is not further metabolized and leaves the cells, while oxaloacetate is decarboxylated to pyruvate by a soluble oxaloacetate decarboxylase encoded by the *mae* gene and termed CitM (25). In a recent study it was demonstrated that in the lactic acid bacterium *Lactococcus lactis* IL1403(pFL3), part of the pyruvate formed from citrate was converted into acetoin and part was converted into acetate in parallel pathways (23). In resting cells, the distribution over the two end products strongly depended on the conditions. A high turnover rate through the pathway favored the formation of acetoin over acetate.

The citrate transporter CitP plays a pivotal role in the kinetics of the pathway. Under physiological conditions,

when citrate is cometabolized with a carbohydrate, CitP operates in the fast citrate/L-lactate exchange mode; citrate is taken up and at the same time that L-lactate, the product of glycolysis, is excreted (precursor/product exchange) (2, 17, 19, 23). In the absence of L-lactate, CitP operates in the much slower unidirectional H⁺-symport mode (19), which makes uptake the rate-determining step for the flux through the pathway (23). It was shown that, in addition to L-lactate, CitP has an affinity for intermediates/products of the pathway, which results in the typical biphasic consumption of citrate in the absence of L-lactate. During the first phase, citrate is taken up slowly in symport with an H⁺, but as intermediates/products accumulate in the cytoplasm, the transporter switches to the fast exchange mode, in which citrate is taken up in exchange with pyruvate, α -acetolactate, and/or acetate, resulting in much higher consumption rates in the second phase. Therefore, the kinetics and product profile of citrate metabolism are determined largely by the availability of exchangeable substrates for the transporter CitP.

The physiological function of citrate metabolism in LAB is in metabolic energy generation and acid stress resistance (8, 18, 20, 28). The citrate metabolic pathway generates proton motive force (PMF) (3, 19, 20) by an indirect proton pumping mechanism, by which the two components of the PMF, membrane potential ($\Delta\psi$) and pH gradient (ΔpH), are generated in separate steps (13, 14). The exchange of divalent citrate and monovalent lactate catalyzed by CitP generates membrane po-

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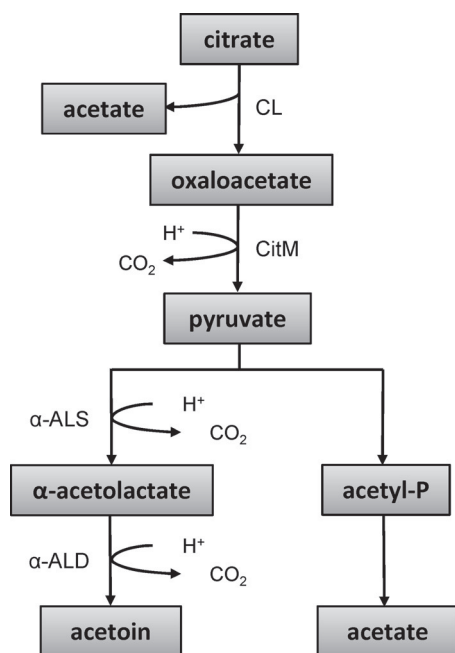


FIG. 1. Schematic representation of citrate metabolism by *L. lactis* IL1403(pFL3). CL, citrate lyase; CitM, oxaloacetate decarboxylase; α -ALS, α -acetolactate synthase; α -ALD, α -acetolactate decarboxylase. The stoichiometry of the reactions was not taken into account.

tential of physiological polarity (i.e., inside negative). In *L. lactis* IL1403(pFL3), the exchange of citrate and the intermediates/products acetate and α -acetolactate was shown to be electrogenic as well (23). Proton consumption in the decarboxylation of oxaloacetate and α -acetolactate, as well as the formation of the latter (Fig. 1), results in a pH gradient (inside alkaline). The pathway from pyruvate to acetate, which has not been resolved completely, does not seem to generate PMF (23). The PMF may be used to produce additional ATP via F_1F_0 -ATPase or to maintain a high cytoplasmic pH to improve the acid resistance of the strain.

A recent study reported the consequences of a null allele of oxaloacetate decarboxylase in *L. lactis* strain ILCitM(pFL3) on growth and intracellular pH maintenance in the presence and absence of citrate (1). While no effect on the maintenance of the cytoplasmic pH was observed, growth was impaired in the presence of citrate, which was correlated with the transient accumulation of oxaloacetate in the medium. Transporters for oxaloacetate are rarely found in bacteria. The dicarboxylate transporters DccT of *Corynebacterium glutamicum* (31) and SdcL of *Bacillus licheniformis* (29) as well as the DctA transporters of *C. glutamicum* (32) and *Bacillus subtilis* (10) were shown to be competitively inhibited by oxaloacetate. In the present study, the physiological response of the oxaloacetate-deficient mutant to the potentially toxic accumulation of oxaloacetate in the cytoplasm is demonstrated. The mechanistic basis of citrate metabolism in the mutant is the ability of the transporter CitP to excrete oxaloacetate in exchange with citrate and to take up oxaloacetate in exchange with L-lactate or another intermediate/product of the pathway.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *Lactococcus lactis* IL1403 (6), IL1403(pFL3) (16), and an oxaloacetate decarboxylase mutant of IL1403(pFL3) named ILCitM(pFL3) (1) were used in this study. Plasmid pFL3 harbors the *citP* gene under the control of the *Streptococcus pneumoniae* *polA* promoter (16). Neither expression nor plasmid copy number is under the control of citrate or pH in strain IL1403(pFL3) (8). Mutant strain ILCitM was constructed from IL1403 by a deletion of 14 bp between positions 584 and 598 of the oxaloacetate decarboxylase gene, the *mae* gene (1). Precultures were grown overnight at 30°C in M17 broth medium supplemented with 0.5% (wt/vol) glucose (M17G) and 5 μ g ml⁻¹ of tetracycline when appropriate. Cells were grown in M17G medium with an initial pH adjusted to 7.0. Growth was performed in 100-ml serum bottles without agitation and at 30°C. Growth was monitored by measuring the optical density at a wavelength of 660 nm (OD₆₀₀). Cells were harvested at the mid-exponential growth phase when the optical density was 0.6 by spinning for 10 min at 3,000 rpm. Cells were washed two times with 50 mM potassium phosphate (pH 5.8) buffer and finally resuspended in the same buffer at 4°C.

Citrate and oxaloacetate consumption by resting cells. Resting cells at an OD₆₀₀ of 1.5 in 50 mM potassium phosphate (pH 5.8) buffer were incubated at 30°C without agitation for 10 min. The assay was performed with a total volume of 1.5 ml. At time zero, citrate or oxaloacetate was added at a concentration of 2 mM. When indicated, 0.1 mM glucose or 0.2 mM L-lactate was added together with citrate. Samples of 100 μ l were taken every 5 or 10 min and immediately centrifuged for 0.5 min at maximum speed in a table-top centrifuge. The supernatant was stored on ice until further analysis by enzymatic assays and/or high-performance liquid chromatography (HPLC). Measurements of the concentrations of citrate, oxaloacetate, and pyruvate were in good agreement between the two methods. Each experiment was done at least in triplicate, and the averages and standard deviations of data from three independent experiments are shown.

Enzymatic assays. Citrate, oxaloacetate, and pyruvate were measured as described previously (23), using the commercially available enzymes citrate lyase (CL), L-malate dehydrogenase (L-MDH), and L-lactate dehydrogenase (L-LDH). Briefly, an aliquot of 30 μ l of the sample was added to 50 mM glycyl-glycine (pH 7.8) buffer containing NADH and L-MDH. Oxaloacetate in the sample was converted to L-malate at the expense of NADH. Subsequently, pyruvate in the same sample was measured by the addition of L-LDH, which resulted in the conversion of pyruvate to L-lactate at the expense of NADH. The subsequent addition of CL converted the citrate in the sample into oxaloacetate (and pyruvate), resulting in an additional decrease in the NADH concentration equivalent to the citrate concentration present in the samples. The assay was performed with 96-well microtiter plates. The decrease in the NADH concentration was measured spectroscopically at 340 nm.

HPLC analysis. Products of citrate metabolism were determined by loading an aliquot of 10 μ l of the supernatant onto an Aminex HPX-87H anion-exchange column (Bio-Rad Laboratories, Inc., Richmond, CA) operated at 30°C in isocratic mode using 0.005 M H₂SO₄ as the mobile phase and a flow rate of 0.8 ml/min. Quantification was based on calibration curves generated by injecting standards of known concentrations.

Measurements of Δ pH and $\Delta\psi$. The components of the proton motive force were measured as described previously (23). To measure the internal pH (Δ pH), resting cells resuspended to a high density (typically containing 50 mg/ml of protein) in 50 mM potassium phosphate (pH 5.8) buffer were loaded with 2',7'-bis-(2-carboxyethyl)-5 (and -6)-carboxyfluorescein (BCECF). Fluorescence measurements were performed with 1-cm cuvettes containing 50 mM potassium phosphate (pH 5.8) buffer equilibrated at 30°C, and cells were loaded with BCECF. The cuvette was stirred with a magnetic stirring bar. Fluorescence was measured by using excitation and emission wavelengths of 502 and 525 nm, respectively, with slit widths of 4 and 16 nm, respectively. The fluorescence signal was sampled every second. The opening of the measurement compartment caused a loss of data during the first 5 to 6 s after an addition to the cuvette was made. The cytoplasmic pH was calculated as described previously (21).

The membrane potential ($\Delta\psi$) was measured qualitatively with the fluorescent probe 3,3'-dipropylthiocarbocyanine iodide (DiSC₃) (26). A decrease in the fluorescence intensity correlates with an increase in the electrical potential across the membrane. DiSC₃ was added from a stock solution to a final concentration of 2 μ M to the quartz cuvettes containing 50 mM potassium phosphate (pH 5.8) buffer and cells. The system was left to equilibrate for 5 min at 30°C. Fluorescence measurements were performed by using excitation and emission wavelengths of 500 and 705 nm, respectively, and slit widths of 8 nm.

Chemicals. L-LDH, L-MDH, and CL were obtained from Roche Applied Science. BCECF (acid form) and DiSC₃ probes were obtained from Invitrogen Molecular Probes.

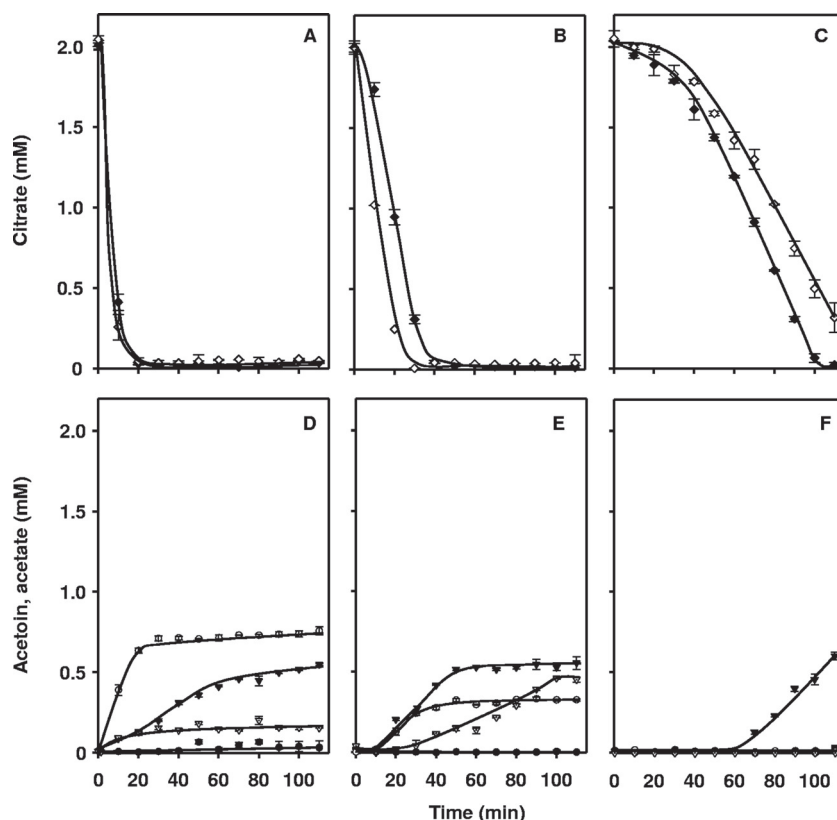


FIG. 2. Citrate consumption and formation of the end products of citrate metabolism in *L. lactis* IL1403(pFL3) and ILCitM(pFL3). Citrate consumption (\diamond and \blacklozenge) and acetoin (\circ and \bullet) and acetate (∇ and \blacktriangledown) formation by resting cells of *L. lactis* IL1403(pFL3) (open symbols) and ILCitM(pFL3) (closed symbols) in the presence of 0.1 mM glucose (A and D), 0.2 mM L-lactate (B and E), and no further additions (C and F). At time zero, a concentration of 2 mM citrate was added to the cell suspensions in 50 mM potassium phosphate (pH 5.8) buffer.

RESULTS

Citrate metabolism by *L. lactis* ILCitM(pFL3). The consumption of citrate and the formation of the end products acetoin and acetate were studied with resting cells of *L. lactis* strains IL1403(pFL3) and ILCitM(pFL3) in the presence and absence of glucose or L-lactate.

Both strains express the citrate transporter CitP in *trans* from a nonnative promoter to decouple *citP* expression from the native control (16). CitP encoded on plasmid pFL3, together with the metabolic enzymes encoded on the chromosome (5), completes the citrate metabolic pathway. Strain ILCitM(pFL3) contains a frameshift deletion of 14 bp in the *mae* gene, resulting in a null allele of the oxaloacetate decarboxylase CitM (Fig. 1) (1). Both strains were grown until the mid-exponential growth phase ($OD_{660} = 0.6$) in M17 broth medium at pH 7.0 supplemented with 0.5% (wt/vol) glucose and in the absence of citrate (23).

Resting cells of strain IL1403(pFL3) resuspended at an OD_{660} of 1.5 in 50 mM KP_i (pH 5.8) buffer rapidly consumed 2 mM citrate in the presence of 0.1 mM glucose (Fig. 2A). Surprisingly, the oxaloacetate-deficient strain ILCitM(pFL3), which is blocked in the citrate metabolic pathway (Fig. 1), showed more or less the same consumption rate. The rates were 0.17 and 0.16 mM/min for the parental and mutant strains, respectively (Table 1). Under this condition in the parental strain, citrate uptake is catalyzed by the transporter

CitP in exchange with L-lactate produced from glucose. The conditions may be mimicked by the addition of L-lactate rather than glucose directly to the buffer (Fig. 2B). L-Lactate, present at a substoichiometric concentration relative to that of citrate (0.2 and 2 mM, respectively), allows CitP to operate in the fast exchange mode via the so-called “shuttle” mechanism. L-Lactate exported from the cells by CitP reenters as L-lactic acid by passive diffusion (19, 23). Similarly to what was observed in the presence of glucose, the rate of citrate consumption in the presence of L-lactate was only marginally lower in the oxaloacetate-deficient mutant than in the parental strain, with rates of 0.07 and 0.09 mM/min, respectively. A short delay was observed for the mutant strain, which was not observed with the parental strain (Fig. 2B). In the absence of L-lactate (or

TABLE 1. Rates of citrate consumption (v) in *L. lactis* IL1403(pFL3) and ILCitM(pFL3) in the presence and absence of glucose and L-lactate

Addition ^a	Mean v (mM/min) \pm SD	
	IL1403(pFL3)	ILCitM(pFL3)
0.1 mM glucose	0.17 \pm 0.02	0.16 \pm 0.01
0.2 mM L-lactate	0.09 \pm 0.01	0.07 \pm 0.01
None	0.025 \pm 0.003 ^b	0.029 \pm 0.003 ^b

^a In addition to 2 mM citrate.

^b Second phase (Fig. 1).

TABLE 2. Product formation from citrate by resting cells of *L. lactis* ILCitM(pFL3) under different conditions measured by HPLC

Condition ^a	Mean concn of product (mM) \pm SD				Mean yield ^c \pm SD
	Oxaloacetate	Pyruvate	Acetate ^b	α -Acetolactate	
0.1 mM glucose					
20 min (citrate depleted)	1.6 \pm 0.01	0.17 \pm 0.04	0.16 \pm 0.05	0.03 \pm 0.01	1.96 \pm 0.11
110 min	0.82 \pm 0.02	0.44 \pm 0.08	0.55 \pm 0.06	0.13 \pm 0.02	1.94 \pm 0.18
0.2 mM L-lactate					
40 min (citrate depleted)	1.71 \pm 0.02	0.14 \pm 0.02	0.12 \pm 0.09	0.02 \pm 0.01	1.99 \pm 0.14
110 min	1.2 \pm 0.02	0.36 \pm 0.04	0.42 \pm 0.08	0.02 \pm 0.02	2 \pm 0.16
No addition					
100 min (citrate depleted)	1.59 \pm 0.01	0.33 \pm 0.06	0	0.06 \pm 0.01	1.98 \pm 0.08

^a In addition to 2 mM citrate. Time points are shown in Fig. 4.

^b In addition to the equimolar concentration of acetate formed by citrate lyase.

^c In equivalents of citrate (2 mM).

glucose), citrate consumption by the parental strain is characterized by two distinct phases (Fig. 2C) (23). During the first slow phase, intermediates and products of the pathway build up in the cytoplasm, which are excreted in exchange for citrate in the second fast phase. The mutant strain showed the same behavior, but the first phase was shorter, and the rate of the second phase was higher (Fig. 2C).

The metabolism of citrate in resting cells of *L. lactis* IL1403(pFL3) was shown previously to result in the production of two end products, acetoin and acetate, depending on the conditions (23). Acetoin was the major product of citrate metabolism when the rate of citrate consumption was high, i.e., in the presence of glucose or L-lactate (0.67 and 0.5 mM, respectively) (Fig. 2D and E). A high rate of citrate consumption results in the accumulation of pyruvate in the cytoplasm, which favors the acetoin synthesis pathway because of the low affinity of α -acetolactate synthase (α -ALS) for pyruvate ($K_m = 50$ mM) (Fig. 1) (27). The kinetics of acetoin formation (one molecule of acetoin is formed from two molecules of citrate) follow the kinetics of citrate degradation. In contrast, under the same conditions and with similar rates of citrate consumption, mutant strain ILCitM(pFL3) did not produce any measurable acetoin, which would be in agreement with the pathway being interrupted following oxaloacetate. Instead, a significant amount of acetate, in addition to the acetate produced by citrate lyase, was produced, suggesting, in contradiction, that the pathway following oxaloacetate was still functional. Acetate production by the mutant was clearly delayed. When all citrate was depleted, the amounts of additional acetate produced were 0.16 mM and 0.12 mM in the presence of glucose and L-lactate, respectively (Table 2). Following the depletion of citrate, the amounts of acetate increased to 0.55 mM and 0.42 mM, respectively (Table 2 and Fig. 2D and E). In the absence of glucose or L-lactate, the parental strain produced acetate in the second phase but no acetoin (23), while neither of the products was detected in the case of the mutant strain during the course of the experiment (Fig. 2F). The experiments demonstrate that the oxaloacetate-deficient mutant metabolized citrate at rates similar to those of the parental strain, but the product profiles were significantly different. While the pathway to acetoin appeared to be blocked, the route to acetate appeared to be functional (Fig. 2).

Energetics of the citrate metabolic pathway in *L. lactis* ILCitM(pFL3). Citrate metabolism in LAB generates metabolic energy by generating PMF. Membrane potential is generated in the citrate uptake step by the electrogenic exchange of divalent citrate and monovalent lactate, while proton consumption in the decarboxylation reactions in the pathway result in a transmembrane pH gradient that is alkaline inside (18, 19, 20). The transmembrane pH gradient was evaluated by measurements of the internal pH inferred from the fluorescent dye BCECF that was trapped inside the cells (21). The membrane potential was measured qualitatively by the potentiometric probe DiSC₃ (26). Previously, it was shown that both probes had an inhibitory effect on the flux through the citrate pathway in strain IL1403(pFL3), which in part could be compensated for by an increase of the L-lactate concentration. The addition of 2 mM citrate in the presence of 1 mM L-lactate resulted in a pH gradient (Δ pH) of 0.7 units that developed during the first 10 min of citrate consumption (Fig. 3A). The kinetics of membrane potential ($\Delta\Psi$) generation were faster. A steady state was reached within 2 min (Fig. 3B). The similar rate of citrate consumption by mutant strain ILCitM(pFL3) (Fig. 2B) did not result in the generation of a Δ pH, and only a small change in the $\Delta\Psi$ was observed (Fig. 3A and B). It should be noted that the measurements reflect steady-state values of PMF obtained at a constant rate of citrate consumption (Fig. 2B). For technical reasons, the methods do not allow measurements over longer periods of time after all citrate is consumed. It follows that at the same high rate of citrate consumption, the generation of proton motive force is severely inhibited in the oxaloacetate decarboxylase-deficient mutant.

Oxaloacetate is excreted during citrate consumption in *L. lactis* ILCitM(pFL3). Citrate metabolism in *L. lactis* IL1403(pFL3) resulted in, in addition to the end products, the appearance of citrate pathway intermediates, i.e., α -acetolactate and/or pyruvate, outside the cells. Oxaloacetate, the first intermediate of citrate breakdown, was found in only trace amounts and only when citrate was metabolized in the absence of glucose or L-lactate (23). In contrast, citrate metabolism in the mutant strain resulted in the production of a large amount of oxaloacetate outside the cells. In the presence of 0.1 mM glucose, 0.2 mM L-lactate, or no further additions, 2 mM citrate resulted in 1.6, 1.71, and 1.59 mM oxaloacetate outside the cells, respectively

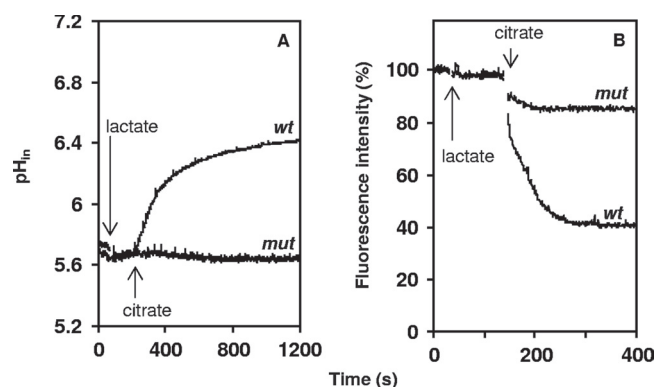


FIG. 3. Energetics of citrate metabolism in *L. lactis* IL1403(pFL3) and ILCitM(pFL3). The internal pH (A) and the membrane potential (B) of cells of *L. lactis* IL1403(pFL3) (wt) and ILCitM(pFL3) (mut) were continuously monitored in time. At the times indicated by the arrows, 1 mM L-lactate and 2 mM citrate were added. The internal pH was evaluated from the pH-dependent fluorescence of BCECF as described in Materials and Methods. Changes in the membrane potential were qualitatively evaluated by the quenching of the potentiometric probe DiSC₃.

(Fig. 4 and Table 2). The increase in the amount of oxaloacetate was kinetically coupled to the decrease in the amount of citrate. Small amounts of pyruvate and/or acetate completed the carbon balance (Table 2). Following the depletion of citrate, the concentration of oxaloacetate outside the cells decreased again (Fig. 4A and B). At the same time, an increase in levels of pyruvate and acetate was observed, suggesting a reuptake of oxaloacetate by the cells and subsequent conversion in the cytoplasm. Oxaloacetic acid is known to be unstable, undergoing spontaneous decarboxylation to pyruvate. Control experiments in buffer demonstrated that under the conditions of the experiment, the nonenzymatic reaction resulted in about 15% of oxaloacetate being converted to pyruvate in 1 h (not shown), which was significantly lower than the decrease in the oxaloacetate concentration observed following the depletion of citrate. The results indicate that in spite of the CitM null allele, the decarboxylation of oxaloacetate in the cytoplasm is

still catalyzed, producing the intermediate pyruvate that in part is excreted by the cells and in part is further metabolized to acetate.

Oxaloacetate is a substrate of CitP. Oxaloacetate is a divalent, negatively charged dicarboxylate that is not likely to cross the cytoplasmic membrane by passive diffusion. The similar kinetics of citrate degradation and oxaloacetate production by mutant strain ILCitM(pFL3) under all conditions tested (Fig. 4) suggest that the excretion of oxaloacetate is coupled to the uptake of citrate by the citrate transporter CitP. Oxaloacetate uptake in parental strain IL1403(pFL3) and in the original strain IL1403, which does not contain the gene encoding the citrate transporter CitP, was studied. In the presence of the latter strain, the concentration of oxaloacetate decreased at a rate that was comparable to that of the spontaneous decarboxylation observed in buffer (Fig. 5B and see above). Apparently, although the cells contain the oxaloacetate decarboxylase enzyme and downstream enzymes for further metabolism, they are unable to take up oxaloacetate from the medium. In contrast, a significantly higher rate of oxaloacetate consumption was observed for strain IL1403(pFL3), containing the citrate transporter CitP. Similar to what was observed with citrate as the substrate (Fig. 2C), the decrease in the concentration of oxaloacetate was biphasic, with very little consumption during the first 10 min. Importantly, the rate of oxaloacetate consumption was enhanced in the presence of L-lactate, consistent with an oxaloacetate/L-lactate exchange catalyzed by CitP. In agreement, L-lactate did not have any effect on the decrease of the oxaloacetate concentration in the presence of strain IL1403 (Fig. 5B). The rate of oxaloacetate consumption by IL1403(pFL3) in the presence of L-lactate decreased as the concentration of oxaloacetate decreased, indicating a lower affinity of CitP for oxaloacetate than that observed for citrate (compare Fig. 2B and 5A).

Oxaloacetate metabolism by *L. lactis* strain IL1403(pFL3) in the presence of 1 mM L-lactate resulted in the generation of a transmembrane pH gradient of 0.5 units (Fig. 6A). Similarly, a small but significant membrane potential was generated (Fig. 6B). Cells of IL1403 lacking the CitP transporter showed no

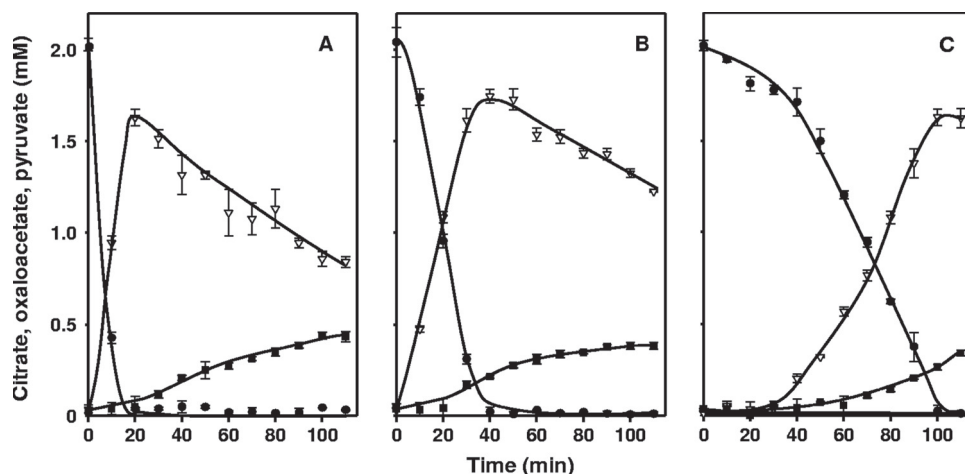


FIG. 4. Products of citrate metabolism in *L. lactis* ILCitM(pFL3). A concentration of 2 mM citrate was added to resting cells of *L. lactis* ILCitM(pFL3) in the presence of 0.1 mM glucose (A), 0.2 mM L-lactate (B), or no further additions (C). Citrate consumption (●) and oxaloacetate (▽) and pyruvate (■) production were measured in time.

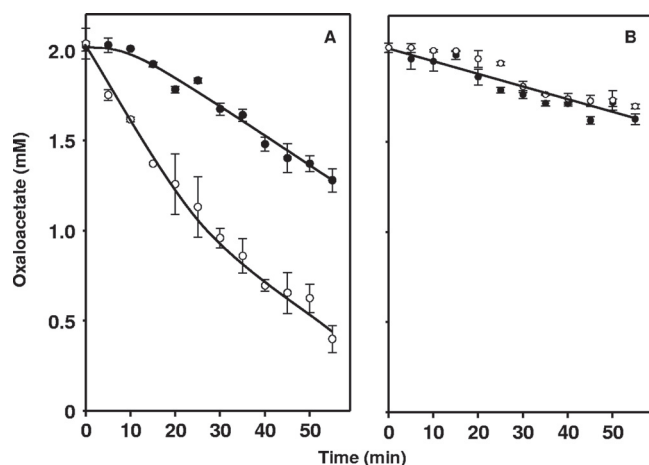


FIG. 5. Oxaloacetate consumption in *L. lactis* IL1403 in the presence and absence of CitP. A concentration of 2 mM oxaloacetate was added to resting cells of *L. lactis* IL1403(pFL3) (A) and IL1403 (B) in the presence (○) and absence (●) of 1 mM L-lactate.

PMF generation upon the addition of oxaloacetate in the presence of L-lactate (Fig. 6). It follows that *L. lactis* IL1403(pFL3) metabolizes oxaloacetate by a mechanism similar to that for citrate.

DISCUSSION

Citrate metabolism in *L. lactis* mutant strain ILCitM(pFL3).

The oxaloacetate-deficient strain ILCitM(pFL3) was grown in batch culture at an initial pH of 7.0 in rich medium (M17) supplemented with 0.5% (wt/vol) glucose as a carbon and energy source and in the absence of citrate. The cells were harvested at the mid-exponential growth phase, where the pH had dropped to 6.8. Under these conditions, fermentation by the parent strain is homolactic (30), and citrate metabolic enzymes are present at levels that give high rates of citrate consumption

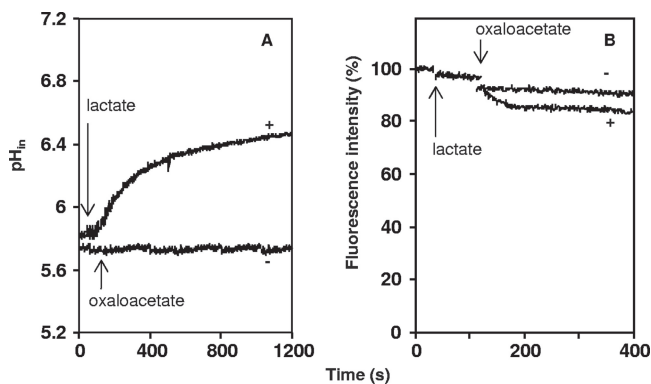


FIG. 6. Energetics of oxaloacetate metabolism in *L. lactis* IL1403 and IL1403(pFL3). The internal pH (pH_{in}) (A) and the membrane potential (B) of cells of *L. lactis* IL1403 lacking CitP (–) and IL1403(pFL3) containing CitP (+) were continuously monitored in time. At the times indicated by the arrows, 1 mM L-lactate and 2 mM oxaloacetate were added. The cells were loaded with BCECF as described in Materials and Methods. Changes in the membrane potential were qualitatively evaluated by the quenching of the potentiometric probe DiSC₃.

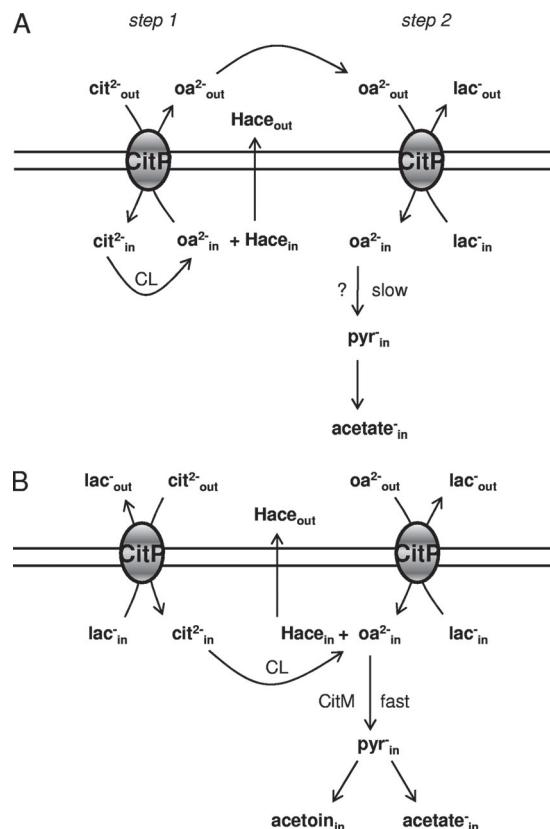


FIG. 7. Transport and metabolism of citrate and oxaloacetate in *L. lactis*. (A) Model of citrate uptake and metabolism in *L. lactis* mutant strain ILCitM(pFL3). (B) Model of citrate or oxaloacetate uptake and metabolism in *L. lactis* parent strain IL1403(pFL3). cit, citrate; oa, oxaloacetate; pyr, pyruvate; ace, acetate; lac, L-lactate.

in resting cells (23). The metabolism of citrate by resting cells of the mutant proceeds in two steps (Fig. 7A). The first step represents a short metabolic pathway in which citrate is converted to oxaloacetate, involving the citrate transporter CitP and citrate lyase (CL). Internalized citrate is converted by citrate lyase, resulting in a rapid accumulation of oxaloacetate in the cytoplasm, which is then excreted in exchange with citrate. This step is omitted in parental strain IL1403(pFL3), as the activity of oxaloacetate decarboxylase prevents the accumulation of oxaloacetate in the cytoplasm. The citrate/oxaloacetate exchange performed by CitP represents a new example of substrate/intermediate exchange, which we previously described (23). The rate of citrate/oxaloacetate exchange is as high as the rate of citrate/L-lactate exchange and higher than the rate of exchange of citrate with other metabolic intermediates/products, which results in a more rapid consumption of citrate by the mutant than that by the parental strain when no L-lactate is present (Fig. 2C). The exchange of divalent citrate and divalent oxaloacetate is electroneutral, and the proton produced in the CL reaction leaves the cell as acetic acid. Hence, the first step does not generate PMF. In the second step, oxaloacetate reenters the cell via CitP in exchange with an available intermediate/product to be slowly converted to the products pyruvate and acetate. No acetoin was formed under the conditions of the experiment, which is consistent with ob-

servations with the parent strain showing that the conversion of pyruvate to acetate rather than acetoin was favored under conditions where the flux through the pathway was low (Fig. 7B) (23). The low rate of the second step is determined by the slow decarboxylation of internalized oxaloacetate (see below). The uptake of divalent oxaloacetate in exchange with a monovalent weak acid, such as L-lactate, pyruvate, or acetate, generates membrane potential, even though this could not be demonstrated in the second phase of citrate consumption due to technical limitations. Together with proton consumption during oxaloacetate decarboxylation, the second step generates PMF.

The oxaloacetate-deficient mutant mimics the physiological conditions of citrate-fermenting *L. lactis* when oxaloacetate accumulates in the cells. Growth experiments showed reduced growth rates of the mutant and an excretion of oxaloacetate into the medium, suggesting toxic effects of high concentrations of the latter in the cytoplasm (1), probably arising from the competitive inhibition of other enzymes. *L. lactis* responds to a lowering of the oxaloacetate degradation rate by the excretion of oxaloacetate in exchange with citrate, thereby making elegant use of the properties of CitP. Once the threat is over, i.e., all citrate is consumed, oxaloacetate is taken up again to be further metabolized slowly. Major consequences are the transient accumulation of oxaloacetate in the external medium, that acetate is the major product, and that the pathway is less efficient in generating PMF since no PMF is generated in the conversion of pyruvate to acetate (23).

Oxaloacetate metabolism in parental strain *L. lactis* IL1403(pFL3). The citrate transporter CitP catalyzes the translocation of both divalent citrate and oxaloacetate across the cytoplasmic membrane in the same transport modes. Fast electrogenic oxaloacetate/L-lactate exchange in the presence of L-lactate (Fig. 5A and 6A) and glucose (not shown) was observed for the wild-type strain. In the absence of L-lactate, oxaloacetate consumption was biphasic, as observed for citrate consumption (Fig. 2C and 5A), indicating uptake in symport with a proton and subsequent exchange with a metabolic intermediate/product. Consequently, cells of *L. lactis* IL1403(pFL3) can metabolize oxaloacetate by a mechanism similar to that for citrate by simply bypassing the citrate lyase reaction (Fig. 7B). Transporters for oxaloacetate are rare in the prokaryotic domains of life. CitP is a member of the 2-hydroxycarboxylate transporter (2HCT) family (TC classification 2.A.24) (28). The 2HCT family is believed to be distantly related to the divalent anion/ Na^+ symporter family (DASS) (TC classification 2.A.47) (15, 24). The dicarboxylate transporters DccT of *Corynebacterium glutamicum* (31) and SdcL of *Bacillus licheniformis* (29) are found in the latter family and were shown previously to be competitively inhibited by oxaloacetate.

Cryptic oxaloacetate decarboxylase activity. In spite of the inactivation of oxaloacetate decarboxylase, oxaloacetate was still decarboxylated in the experiments with the mutant strain. Oxaloacetate has long been known to be unstable in aqueous solution and to spontaneously decarboxylate to yield pyruvate (12). The rate of spontaneous decarboxylation in the buffers used in the present experiments was about 15% per hour, which was too low to account for the oxaloacetate consumption

rate by the mutant strain (Fig. 5). Also, the presence of the cells *per se* did not increase the rate, as the same rate was observed in the presence of wild-type strain IL1403, which does not consume oxaloacetate (Fig. 5B). It follows that the oxaloacetate decarboxylation activity observed for *L. lactis* IL1403(pFL3) must be enzyme catalyzed.

Two classes of oxaloacetate decarboxylases are distinguished: (i) the membrane-bound sodium pumps termed OAD that have been well characterized in Gram-negative bacteria (9) and (ii) the less-well-characterized soluble enzymes from the large malic enzyme (ME) family that are found mostly in Gram-positive bacteria. Oxaloacetate decarboxylase of *L. lactis* encoded by the *mae* gene belongs to the second group, and no OAD genes are carried on the chromosome. The *mae* gene product of *L. lactis* is the only soluble oxaloacetate decarboxylase that has been characterized in detail (25). The deletion mutant was created by a frameshift deletion of 14 bp between positions 584 and 598 in the gene (1). It is highly unlikely that the remaining gene product would fold into a stable enzyme that could account for the low oxaloacetate decarboxylation activity observed for the mutant strain. Most likely, the activity is catalyzed by some other enzyme(s) in the cells as an unknown side reaction. The oxaloacetate decarboxylase activity of other enzymes has been reported. Examples are pyruvate kinase (7) and some MEs (22) that are found to be encoded on the chromosome of *L. lactis* IL1403 (5).

Substrate specificity of CitP. Previous studies performed with right-side-out membrane vesicles derived from *L. lactis* producing CitP of *Leuconostoc mesenteroides* showed that CitP has an affinity for a wide range of physiological and nonphysiological 2-hydroxycarboxylates, all sharing the motif $\text{HO-CR}_2\text{-COO}^-$. Substrates with the motif were all good substrates; however, some 2-ketoacids, i.e., oxaloacetate and pyruvate, and 3-hydroxycarboxylates were also transported by CitP with low efficiencies (2, 4). The assay used in these studies tested the capacity of different substrates to chase accumulated citrate from the membranes and, therefore, for transport from “out” to “in.” More recently, the ability of CitP to translocate efficiently in exchange with the 2-ketoacid pyruvate and the acid acetate was demonstrated by using a different experimental setup with whole cells which assayed transport in the opposite direction, from in to out (“shuttle” mechanism) (23). The transport of acetate was not observed with the previous assay using right-side-out membranes, suggesting that the translocation by CitP in both directions is kinetically asymmetric. The assay employing whole cells requires that the substrates diffuse across the cytoplasmic membrane passively, which limits the assay to monocarboxylates that are permeable in the protonated state. The present study demonstrates that the dicarboxylate 2-ketoacid oxaloacetate is a substrate of CitP as well. The high rate of exchange with citrate by the mutant strain (step 1) (Fig. 7A) shows efficient translocation from in to out. The consumption of oxaloacetate by the parental strain shows that the affinity for transport in the out-to-in direction (step 2) is significantly lower than that for the tricarboxylate 2-hydroxy substrate citrate. It follows that CitP is a remarkable promiscuous transporter with affinity for mono-, di-, and tricarboxylates substituted or not at the C_2 atom.

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REFERENCES

- Augagneur, Y., D. Garmin, and J. Guzzo. 2008. Mutation of the oxaloacetate decarboxylase gene of *Lactococcus lactis* subsp. *lactis* impairs the growth during citrate metabolism. *J. Appl. Microbiol.* **104**:260–268.
- Bandell, M., V. Ansanay, N. Rachidi, S. Dequin, and J. S. Lolkema. 1997. Membrane potential generating malate (MleP) and citrate (CitP) transporters of lactic acid bacteria are homologous proteins. *J. Biol. Chem.* **272**:18140–18146.
- Bandell, M., et al. 1998. Mechanism of the citrate transporters in carbohydrate and citrate cometabolism in *Lactococcus* and *Leuconostoc* species. *Appl. Environ. Microbiol.* **64**:1594–1600.
- Bandell, M., and J. S. Lolkema. 1999. Stereoselectivity of the membrane potential-generating citrate and malate transporters of lactic acid bacteria. *Biochemistry* **38**:10352–10360.
- Bolotin, A., et al. 2001. The complete genome sequence of the lactic acid bacterium *Lactococcus lactis* ssp. *lactis* IL1403. *Genome Res.* **11**:731–753.
- Chopin, A., M. C. Chopin, A. Moillo-Batt, and P. Langella. 1984. Two plasmid-determined restriction and modification systems in *Streptococcus lactis*. *Plasmid* **11**:260–263.
- Creighton, D. J., and I. A. Rose. 1976. Studies on the mechanism and stereochemical properties of the oxalacetate decarboxylase activity of pyruvate kinase. *J. Biol. Chem.* **251**:61–68.
- García-Quintáns, N., C. Magni, D. de Mendoza, and P. López. 1998. The citrate transport system of *Lactococcus lactis* subsp. *lactis* biovar diacetylactis is induced by acid stress. *Appl. Environ. Microbiol.* **64**:850–857.
- Granjon, T., et al. 2010. Structure-function relations in oxaloacetate decarboxylase complex. Fluorescence and infrared approaches to monitor oxomalonate and Na(+) binding effect. *PLoS One* **5**(6):e10935.
- Groeneveld, M., R. G. Weme, R. H. Duurkens, and D. J. Slotboom. 2010. Biochemical characterization of the C₄-dicarboxylate transporter DctA from *Bacillus subtilis*. *J. Bacteriol.* **192**:2900–2907.
- Hugenholtz, J. 1993. Citrate metabolism in lactic acid bacteria. *FEMS Microbiol. Rev.* **12**:165–178.
- Krebs, H. A. 1942. The effect of inorganic salts on the ketone decomposition of oxaloacetic acid. *Biochem. J.* **36**:303–305.
- Lolkema, J. S., B. Poolman, and W. N. Konings. 1995. Role of scalar protons in metabolic energy generation in lactic acid bacteria. *J. Bioenerg. Biomembr.* **27**:467–473.
- Lolkema, J. S., B. Poolman, and W. N. Konings. 1996. Secondary transporters and metabolic energy generation, p. 229–260. In W. N. Konings, H. R. Kaback, and J. S. Lolkema (ed.), *Handbook of biological physics*. Elsevier, Amsterdam, The Netherlands.
- Lolkema, J. S., and D. J. Slotboom. 2003. Classification of 29 families of secondary transport proteins into a single structural class using hydropathy profile analysis. *J. Mol. Biol.* **327**:901–909.
- Magni, C., F. Lopez de Felipe, F. Sesma, P. López, and D. de Mendoza. 1994. Citrate transport in *Lactococcus lactis* subsp. *lactis* biovar diacetylactis. Expression of the citrate permease. *FEMS Microbiol. Lett.* **118**:78–82.
- Magni, C., P. López, and D. de Mendoza. 1996. The properties of citrate transport catalyzed by CitP of *Lactococcus lactis* ssp. *lactis* biovar diacetylactis. *FEMS Microbiol. Lett.* **142**:265–269.
- Magni, C., D. de Mendoza, W. N. Konings, and J. S. Lolkema. 1999. Mechanism of citrate metabolism in *Lactococcus lactis*: resistance against lactate toxicity at low pH. *J. Bacteriol.* **181**:1451–1457.
- Marty-Teyssset, C., J. S. Lolkema, P. Schmitt, C. Divies, and W. N. Konings. 1995. Membrane potential-generating transport of citrate and malate catalyzed by CitP of *Leuconostoc mesenteroides*. *J. Biol. Chem.* **270**:25370–25376.
- Marty-Teyssset, C., et al. 1996. Proton motive force generation by citrolactic fermentation in *Leuconostoc mesenteroides*. *J. Bacteriol.* **178**:2175–2185.
- Molenaar, D., T. Abee, and W. N. Konings. 1991. Continuous measurement of the cytoplasmic pH in *Lactococcus lactis* with a fluorescence pH indicator. *Biochim. Biophys. Acta* **1115**:75–83.
- Park, S. H., B. G. Harris, and P. F. Cook. 1986. pH dependence of the kinetic parameters for the oxalacetate decarboxylation and pyruvate reduction reactions catalyzed by malic enzyme. *Biochemistry* **25**:3752–3759.
- Pudlik, A. M., and J. S. Lolkema. 2011. Citrate uptake in exchange with intermediates of citrate metabolic pathway in *Lactococcus lactis* IL1403. *J. Bacteriol.* **193**:706–714.
- Saier, M. H., Jr. 2000. A functional-phylogenetic classification system for transmembrane solute transporters. *Microbiol. Mol. Biol. Rev.* **64**:354–411.
- Sender, P. D., M. G. Martín, S. Peirú, and C. Magni. 2004. Characterization of an oxaloacetate decarboxylase that belongs to the malic enzyme family. *FEBS Lett.* **570**:217–222.
- Sip, M., J. Herman, J. Plasek, and V. Hrouda. 1990. Transmembrane potential measurement with carbocyanine dye diS-C₃(5): fast fluorescence decay studies. *J. Photochem. Photobiol. B Biol.* **4**:321–328.
- Snoep, J. L., M. J. Teixeira de Mattos, M. J. Starrenburg, and J. Hugenholtz. 1992. Isolation, characterization, and physiological role of the pyruvate dehydrogenase complex and alpha-acetolactate synthase of *Lactococcus lactis* subsp. *lactis* bv. diacetylactis. *J. Bacteriol.* **174**:4838–4841.
- Sobczak, I., and J. S. Lolkema. 2005. The 2-hydroxycarboxylate transporter family: physiology, structure, and mechanism. *Microbiol. Mol. Biol. Rev.* **69**:665–695.
- Strickler, M. A., J. A. Hall, O. Gaiko, and A. M. Pajor. 2009. Functional characterization of a Na⁺-coupled dicarboxylate transporter from *Bacillus licheniformis*. *Biochim. Biophys. Acta* **1788**:2489–2496.
- Thomas, T. D., K. W. Turner, and V. L. Crow. 1980. Galactose fermentation by *Streptococcus lactis* and *Streptococcus cremoris*: pathways, products, and regulation. *J. Bacteriol.* **177**:6928–6936.
- Youn, J. W., E. Jolkver, R. Krämer, K. Marin, and V. F. Wendisch. 2008. Identification and characterization of the dicarboxylate uptake system DccT in *Corynebacterium glutamicum*. *J. Bacteriol.* **190**:6458–6466.
- Youn, J. W., E. Jolkver, R. Krämer, K. Marin, and V. F. Wendisch. 2009. Characterization of the dicarboxylate transporter DctA in *Corynebacterium glutamicum*. *J. Bacteriol.* **191**:5480–5488.